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High-Frequency Genetic Recombination and Reactivation of Orthopoxviruses from DNA Fragments Transfected into Leporipoxvirus-Infected Cells

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Poxvirus DNA is not infectious because establishing an infection requires the activities of enzymes packaged in the virion. This barrier can be overcome by transfecting virus DNA into cells previously infected with another poxvirus, since the resident virus can provide the *trans*-acting systems needed to reactivate transfected DNA. In this study we show that cells infected with a leporipoxvirus, Shope fibroma virus (SFV), can reactivate vaccinia virus DNA. Similar heterologous packaging systems which used fowlpox-infected cells to reactivate vaccinia virus have been described, but SFV-infected cells promoted a far more efficient reaction that can produce virus titers exceeding 10^6 PFU/μg of transfected DNA. SFV-promoted reactions also exploit the hyperrecombinogenic systems previously characterized in SFV-infected cells, and these coupled recombination and reactivation reactions could be used to delete nonessential regions of the vaccinia virus genome and to reconstruct vaccinia virus from overlapping DNA fragments. SFV-catalyzed recombination reactions need only two 18- to 20-bp homologies to target PCR amplicons to restriction enzyme-cut vaccinia virus vectors, and this reaction feature was used to rapidly clone and express a gene encoding fluorescent green protein without the need for plaque purification or selectable markers. The ability of SFV-infected cells to reactivate fragments of vaccinia virus was ultimately limited by the number of recombinational exchanges required and one cannot reconstruct vaccinia virus from multiple PCR fragments spanning essential portions of the genome. These observations suggest that recombination is an integral part of poxvirus reactivation reactions and provide a useful new technique for altering the structure of poxvirus genomes.

Poxviruses are very large DNA viruses that replicate in the cytoplasm of infected cells. Because of interest in the poxvirus variola virus as the causative agent of smallpox, poxvirus research has a long history, dating back the beginnings of modern virology. Some of the earliest experiments described a process called "nongenetic reactivation," wherein cells infected by one poxvirus can promote the recovery of a second virus rendered noninfectious on its own by heat, UV light, or other treatment (8, 15). A characteristic feature of this reaction is that the two viruses need not be genetically identical; for example, vaccinia virus will reactivate variola virus, and myxoma virus will reactivate rabbit fibroma virus. Although the process of nongenetic reactivation has never been characterized in molecular detail, it is generally assumed that the helper virus provides the enzymatic machinery necessary to uncoat, transcribe, repair, and perhaps replicate the inactivated virus, complementing in *trans* other virion components inactivated by heat or other treatments.

Subsequent experiments have shown that replicating poxviruses can also reactivate poxviruses from transfected virus DNA, and several applications of the process which facilitate the production of recombinant viruses have been described. Sam and Dumbell originally demonstrated that one orthopoxvirus could be used to reactivate the DNA of a second virus in a "homologous" packaging reaction (15). Scheiflinger et al. subsequently showed that cells infected with fowlpox virus could reactivate transfected vaccinia virus DNA in a "heterologous" packaging scheme and exploited the narrow host range of fowlpox virus to simplify the rescue and packaging of vaccinia virus recombinants prepared in vitro by DNA ligation (16). Although the method is elegant and has been used in other studies (2, 9, 10), this approach produced recombinant chimeras at efficiencies of only 6 to 14%, and the added technical complexities associated with propagating fowlpox virus have seemingly limited its widespread adoption. A recent publication suggests ways in which the efficiency can be enhanced substantially through the use of a psoralen-inactivated helper virus (19), although this homologous packaging reaction risks recombination between two vaccinia virus genomes, one of which has been subjected to highly mutagenic treatment.

In most of these studies, some care seems to have been taken to extract and restrict virus DNA in ways that minimize shearing of the 190-kbp vaccinia virus genome. Yet no matter how carefully this is done, it is difficult to imagine poxvirus DNA surviving the transfection process intact, and thus the reactivation process presumably repairs transfected viral DNA with the recombination systems readily detected in poxvirus-infected cells. This raises questions concerning the role of recombination in poxvirus reactivation reactions.

In this communication, we show that replicating poxviruses can exploit viral recombination reactions to produce simple recombinants from mixtures of cotransfected virus and PCR-amplified DNAs as well as more complex recombinants from

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multiple overlapping fragments of virus DNA. The reaction products can then be packaged into infectious particles. Our observations suggest that with an efficient heterologous poxvirus reactivation reaction in conjunction with virus recombination reactions, one can genetically manipulate the structure of poxvirus genomes in ways not previously appreciated. This work also provides further insights into the hyperrecombinogenic intracellular environment created by replicating leporipoxviruses.

MATERIALS AND METHODS

Viruses and cell culture. Vaccinia virus strain WR, SFV strain Kasza, myxomavirus strain Lausanne and rabbit SIRC cells were originally obtained from the American Type Culture Collection. Vaccinia virus strain Copenhagen was obtained from N. Scollard (Aventis-Pasteur Canada), vaccinia virus strain VTF7.5 was obtained from P. Traktman (Medical College of Wisconsin), and modified vaccinia virus strain Ankara bearing a *lacZ* insertion [MVA LZ (18)] was obtained from J. Bramson (McMaster University). BSC-40 cells were obtained from E. Niles (SUNY Buffalo), BGMK cells were obtained from G. McFadden (University of Western Ontario), and BHK-21 cells were obtained from Bramson. All cells were propagated at 37°C in 5% CO_2 in minimal essential medium supplemented with L-glutamine, nonessential amino acids, antibiotics, antimycotics, and 5 to 10% fetal calf serum (Cansera). SFV and myxoma viruses were propagated on SIRC cells, and most vaccinia viruses were propagated on BSC-40 cells. MVA LZ was propagated on BHK-21 cells.

Recombinant virus construction. Vaccinia virus strain XY-I-*Sce*IVV was constructed by standard methods. Briefly, pTM3 (3, 11) was digested with *Nco*I and *Xho*I, and the excised polylinker was replaced with a 44-bp oligonucleotide adaptor encoding the I-*Sce*I site (underlined) (5′-CATGGTAGGGATAACAGGGTAATGTGCACCATCACCACCACCAC-3′ and 5′-TCGAGTGGTGGTGGTGATGGTGCACATTACCCTGTTATCCCTAC-3′). The resulting plasmid (pXY-I-*Sce*I) was purified and partially sequenced to confirm the insert structure, and calcium phosphate was used to transfect the DNA into vaccinia virus-infected BSC-40 cells. Recombinant gpt^+ viruses were passaged three times and plaque purified twice with mycophenolic acid selection. Southern blots were used to confirm the structure of the selected recombinant virus and to confirm that the introduced site could be cut by I-*Sce*I.

Virus reactivation assays and DNA transfection methods. BGMK cells were grown to near confluency in 60-mm dishes and then infected with SFV at a multiplicity of infection of 1 to 2 for 1 h at room temperature in 0.5 ml of phosphate-buffered saline. The buffer was replaced with 3 ml of warmed growth medium, and the cells were returned to the incubator for another hour. Lipofectamine complexes were prepared by mixing 2 to 5 μg of vaccinia virus DNA in 0.5 ml of Optimem medium with diluted Lipofectamine LF2000 reagent (6 to 15 μl of Lipofectamine plus 0.5 ml of Optimem medium). The mixture was incubated for 20 min at room temperature, and then 1 ml was added to each dish of cells and incubated for another 4 h at 37°C in a CO_2 incubator. The transfection solution was replaced with 5 ml of fresh growth medium, and the cells were cultured another 3 to 4 days at 37°C. Virus particles were recovered by scraping the cells into the culture medium and subjecting the mix to three cycles of freezing and thawing. This crude extract was diluted 10^1- to 10^5-fold in phosphate-buffered saline and plated on BSC-40 cells to recover vaccinia virus. Plaques were stained with a solution containing either 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), to detect recombinant β-galactosidase activity, or with Giemsa or crystal violet stain, to titrate total virus.

Other DNAs. Vaccinia virus particles were purified by sedimentation through sucrose gradients, and then the DNA was recovered and purified by proteinase K digestion, phenol extraction, and ethanol precipitation. A commercial pulsed-field gel electrophoresis system and 1% agarose gels were used as directed by the manufacturer (Bio-Rad) to size fractionate vaccinia virus DNAs. Gene targeting experiments used a number of different β-galactosidase gene cassettes prepared with the PCR and several different primer pairs. A high-fidelity DNA polymerase (Expand high-fidelity PCR system; Roche) was used as directed by the manufacturer. The template was plasmid pTKZ-1, which encodes the *Escherichia coli* β-galactosidase gene regulated by a vaccinia virus 7.5S promoter (17).

DNAs designed to target the endogenous *Not*I site in wild-type vaccinia virus were prepared with the two 37-mer primers pTKZ1-LacZ*Not*I18-A (5′-ACACCGACGATGGCGGCCCTTAAAAATGGATGTTGTG-3′) and pTKZ1-LacZ*Not*I18-B (5′-TTCGTGTCTGTGGCGGCCCCTCAAAATACATAAACGG-3′). This created a targeting cassette sharing two repeats of 18 bp of flanking homology with *Not*I-cut virus. To prepare inserts targeting the I-*Sce*I site in virus XY-I-*Sce*IVV, we PCR amplified the insert with the 37-mer primers pTKZ1-LacZ-A (5′-GATAATACCATGGTAGGGCTTAAAAATGGATGTTGTG-3′) and pTKZ1-LacZ-B (5′-ATGGTGCACATTACCCTGCCTCAAAATACATAAACGG-3′) or the 69-mer primers pTKZ1-LacZ-A50 (5′-CCACGGGGACGTGGTTTTCCTTTGAAAAACACGATAATACCATGGTAGGGCTTAAAAATGGATGTTGTG-3′) and pTKZ1-LacZ-B50 (5′-TAATTAATTAGGCCTCTCGAGTGGTGGTGGTGATGGTGCACATTACCCTGCCTCAAAATACATAAACGG-3′). This created DNA cassettes sharing two 18-bp (7.5KZ18) or two 50-bp (7.5KZ50) repeats of flanking homology with *Sce*I-cut XY-I-*Sce*IVV, respectively. A similar approach was used to target an open reading frame encoding enhanced green fluorescent protein (GFP) to the same I-*Sce*I locus. In this case the gene was PCR amplified with the primers GFP-*Sce*I20A (5′-ACGATAATACCATGGTAGGGATGGTGAGCAAGGGCGAGGA-3′) and GFP-*Sce*I20B (5′-TGATGGTGCACATTACCCTGTTACTTGTACAGCTCGTCCA-3′) and a pEGFP-N1 template (Clontech).

In addition to these substrates, a series of long overlapping PCR fragments spanning nearly all of the vaccinia virus genome were prepared with the primer pairs summarized in Table 1. A number of different thermoresistant DNA polymerases were tested for use in this application. The Roche Expand long template PCR kits was eventually found to most reliably amplify long PCR fragments. The DNA sequence of vaccinia virus strain Copenhagen (GenBank entry M35027) and a draft sequence of vaccinia virus strain WR (kindly provided by B. Moss, National Institutes of Health) were used in primer design work. These and other PCR-amplified DNAs were gel purified and electroeluted before use. Spectrophotometry was used to calculate all of the DNA concentrations prior to transfection.

Confocal microscopy. The production of GFP by recombinant viruses was detected with a Leica TCS SP2 confocal microscope. BSC-40 cells were cultured on glass slides, coinfected with a mixture of reactivated recombinant vaccinia virus and a vaccinia virus expressing T7 RNA polymerase (VTF7.5), and imaged 24 h postinfection. The expression of GFP was detected by epifluorescence, while cells were imaged with differential interference contrast optics.

RESULTS

Reactivation of vaccinia virus by Shope (rabbit) fibroma virus. The leporipoxvirus Shope fibroma virus (SFV) and the orthopoxvirus vaccinia virus offer several attractive biological features that simplify the experimental approach that follows. In particular, SFV has a very narrow host range, replicating only in rabbit cells and a few selected monkey cells (BGMK). It also grows slowly to modest titers (≈10^7 PFU/ml), and the minute (≈1-mm) plaques look much like transformed foci. In contrast, vaccinia virus has a much broader host range than SFV, grows rapidly to high titers (≈10^9 PFU/ml), and produces large and distinctive cytolytic plaques. As with previously described vaccinia virus-fowlpox systems, these phenotypic properties greatly facilitate the separation and differentiation of mixtures of SFV and vaccinia viruses.

We infected BGMK cells with SFV, and 2 hours later we transfected these cells with 2 to 5 μg of DNA extracted from sucrose gradient-purified particles of vaccinia virus strain XY-I-*Sce*IVV. Three days posttransfection, all of the infectious particles were recovered by cell lysis and replated on the BSC-40 cell line, which supports the growth only of vaccinia virus. The resulting stained dishes are shown in Fig. 1. Large amounts of virus were recovered with this strategy (yields ranged up to 10^7 PFU/dish of transfected cells), and the plaques visually resembled those produced by the parent strain of vaccinia virus.

Strain XY-I-*Sce*IVV encodes a *gpt* selectable marker, and the reactivated viruses also plated efficiently in the presence of mycophenolic acid (74% of the plaques recovered in the absence of selection). The limit of sensitivity was <20 PFU/ml; within this experimental constraint, no plaques were detected

TABLE 1. PCR primers used to amplify overlapping fragments of the vaccinia virus genome[a]

Amplicon	Size (kbp)	Primer no.	Primer sequence	Position (WR)
PCR12	11.9	25VV5533U18	AGTTAGTTCCGACGTTGA	4900
		26VV19848L21	TATTTGTTGGCTCAGTATGAC	16791
PCR13	11.9	29VV14300U22	TATCAGATTATGCGGTCCAGAG	7458
		30VV22471L21	TGTACTATTCCGTCACGACCC	19411
PCR1	15.1	31VV20807U24	AGCAAGTAGATGATGAGGAACCAG	18727
		32VV36885L22	AGGCAGAGGCATCATTTTGGAC	33836
PCR2	18.2	3VV28266U18	TTAGTTATTTCGGCATCA	25217
		4VV46527L21	TTAGTATTTCTACGGGTGTTC	43416
PCR3	17.3	5VV44435U21	AGAATATCCCAATAGGTGTTC	41306
		6VV61698L20	CTGTTATTATCGACGAGGAC	58586
PCR4	18.6	7VV61397U21	CATTATCTATATGTGCGAGAA	58266
		8VV80029L17	TGACGGGAACAGTAGAA	76914
PCR4L	21.3	7VV61397U21	CATTATCTATATGTGCGAGAA	58266
		8VV79532L29	GATAACCATGTTCTTATTCTTTCTCCTAC	79532
PCR5	19.8	9VV78408U18	AAATGTAGACTCGACGGA	75277
		10VV98171L21	ATAACATATCGACGACTTCAC	95046
PCR6	16.5	11VV96083U20	CATAGAAATAAGTCCCGATG	92938
		12VV112600L21	ATGATATTTCTATTGGCCTAA	109475
PCR7	17.9	13VV111111U19	AGATCGCTTTCTGGTAACA	107972
		14VV129024L21	TTGCCTCTTACTAGCTTAGTT	125916
PCR8	22.3	15VV128103V20	AAGTAGACATAGCCGGTTTC	124975
		16VV146278L21	GTTTATCTTTACGGGCATTAC	147319
PCR9	19.2	17VV145376U21	ATGTCCTCTGCCAAGTACATA	146382
		18VV164550L20	AGTACATTATTCACGCTGTC	165581
PCR10	15.3	19VV159718U21	TATATTCTTTCAACCGCTGAT	160733
		20VV175026L19	AACCGGGATGTAATAACAC	176016
PCR11	20.5	23VV169321U21	TGCCATTATGATAAGTACCCT	170316
		24VV187184L21	TGTCTTTCTCTTCTTCGCTAC	190831

[a] Except for primer 8VV79532L29, the primer no. specifies the position within the vaccinia virus (Copenhagen) genome. For example, the 5′ end of primer 25VV5533U18 maps to nucleotide position 5533. Primer 8VV79532L29 is located at position 82662 in strain Copenhagen.

when vaccinia virus DNA was transfected into uninfected cells, nor were any cytolytic plaque-forming particles recovered from cells infected only with SFV. Microscopic inspection of the control dishes also failed to detect any plaques resembling the foci formed by SFV, although we could not preclude the possibility that SFV establishes an abortive infection in BSC-40 cells. To prove that the method produces bona fide vaccinia viruses, we plaque purified several independent virus isolates, extracted virus DNA, and used Southern blots to compare the *Hin*dIII fingerprint of each isolate with that of the parent vaccinia virus strain, XY-I-*Sce*IVV, and its precursor strain, vaccinia virus WR. Figure 2 illustrates one such Southern blot; all of the rescued viruses appeared identical to the parent strain at this level of resolution.

Reciprocal reactivation of leporipoxviruses. We also tested whether the reciprocal experiment would work, that is, can an orthopoxvirus reactivate a leporipoxvirus? We took advantage of the narrow host range of modified vaccinia virus strain Ankara to test whether MVA could reactivate myxoma virus. (Myxoma virus was used in these experiments because it produces more easily visualized and accurately titered plaques than does SFV.) Preliminary tests showed that both viruses replicated efficiently on hamster BHK-21 and monkey BGMK cells, but only myxoma virus produced plaques on rabbit SIRC cells. We infected BGMK cells with a *lacZ*$^+$ derivative of MVA [MVA LZ (18)] and then transfected the cells with wild-type myxoma virus DNA. Four days later the resulting viruses were recovered and plated on SIRC cells. Viruses were recovered with yields of ≈300 PFU/μg of transfected DNA, and none of these plaques stained positively for the *lacZ* marker characteristic of MVA LZ or *lacZ*$^+$ intertypic recombinants. Thus, it would seem that although the reaction is less efficient, if one uses the appropriate selection strategy an orthopoxvirus can reactivate a leporipoxvirus.

Genetic recombination is associated with virus reactivation. The vaccinia virus genome spans 196 kbp, and no special efforts were made to avoid shearing viral DNA during the process of DNA extraction. Pulsed-field gels showed that the double-stranded DNA used in these experiments contained the expected distribution of broken molecules ranging in size from <10 kbp to near full length (Fig. 3, lane 2). We have shown previously (21) that poxvirus-infected cells catalyze high-frequency recombination of transfected DNAs with a single-strand annealing mechanism, and we presumed that SFV-infected cells catalyze recombinational repair of this sheared vaccinia virus DNA in much the same way in reactivation reactions.

To examine this question in more detail, we separately digested purified wild-type vaccinia virus DNA with *Bss*HII and *Sac*II and examined the ability of SFV-infected cells to reconstruct intact genomes and live viruses from these linearized fragments. Pulsed-field gels showed that these enzymes cut vaccinia virus strain WR DNA to completion (Fig. 3), and the restriction fragments, with some strain-specific exceptions, closely matched those predicted by computational methods (Fig. 4). When these DNAs were transfected separately into SFV-infected BGMK cells, they produced no recombinant vaccinia viruses detectable by plating on BSC-40 cells (<20 PFU/dish). However, cotransfecting a mixture of *Sac*II- and *Bss*HII-cut DNAs into SFV-infected cells permitted the production of

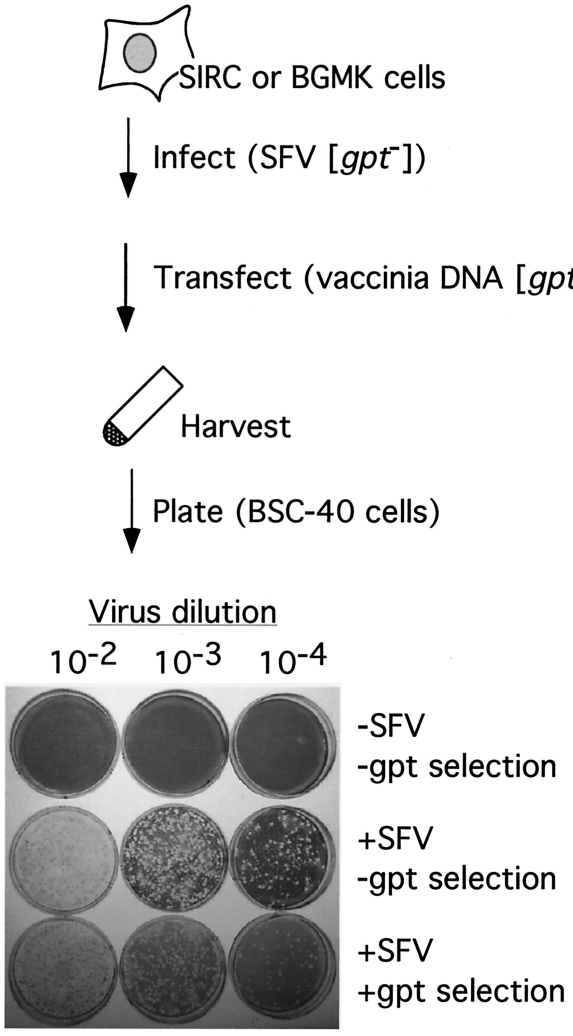

FIG. 1. Reactivation of vaccinia virus DNA in SFV-infected cells. BGMK cells were infected with SFV for 1 h at a multiplicity of infection of 2, and 1 h later they were transfected with 5 μg of purified vaccinia virus XY-I-*Sce*IVV DNA in Lipofectamine LF2000. The cells were cultured for 3 days at 37°C, any resulting virus particles were recovered by freeze-thawing, and different virus dilutions were plated on a BSC-40 cell line to select for growth of vaccinia virus. Some of the viruses were also plated in the presence of mycophenolic acid to select for the gpt^+ selectable marker encoded by strain XY-I-*Sce*IVV. The plates of BSC-40 cells shown here were stained with Giemsa dye 3 days postinfection and exhibited a characteristic pattern of cytolytic plaques typical of vaccinia virus. No vaccinia viruses were recovered from mock-infected and transfected cells or from cells infected only with SFV.

infectious vaccinia virus particles at levels essentially identical to the control, uncut, reaction efficiency (2.5×10^5 versus 2.6×10^5 PFU/dish). Similarly, cotransfecting SFV-infected cells with an equimolar mixture of two large gel-purified *Bgl*I-A and *Stu*I-A restriction fragments (Fig. 4) also permitted the recovery of recombinant viruses (2×10^3 PFU/dish). Nevertheless, there are limits to these reactions. Attempts to reconstruct vaccinia virus from a mixture of *Hin*dIII- and *Xho*I-cut molecules were unsuccessful, suggesting that such enzymes probably cut too frequently or too close to each other to preclude the reassembly of intact vaccinia virus genomes by SFV-infected cells.

Production of recombinant viruses by targeted double-strand break repair. Targeted double-strand break repair can be exploited to simplify the construction and recovery of recombinant vaccinia viruses without plasmid cloning or DNA ligation reactions. Vaccinia virus was modified by standard molecular biological and plasmid-by-virus recombination methods to incorporate an I-*Sce*I site and *Escherichia coli gpt* selectable marker into the thymidine kinase gene locus (strain XY-I-*Sce*I; Fig. 5). Virus DNA was then isolated from purified XY-I-*Sce*I particles, digested with I-*Sce*I, and cotransfected along with a 20-fold molar excess of a PCR-amplified β-galactosidase gene cassette into SFV-infected cells (Fig. 5). In this case, the β-galactosidase gene was placed under the regulation of a 7.5S promoter, and the PCR amplicon incorporated two 18-bp end sequences identical to sequences flanking the recombinant I-*Sce*I site.

X-Gal staining showed that this approach produced about 30% recombinant viruses, and Southern blots confirmed that all of the putative recombinants tested (10 of 10) arose through the expected targeted recombination between the β-galactosidase gene and the I-*Sce*I cleavage site (Fig. 5). Subsequent experiments showed that the frequency of recombinant production was enhanced by increasing the ratio of insert to virus vector and by increasing the length of terminal homology. Cells cotransfected with I-*Sce*I-cut vaccinia virus DNA and a 40-fold excess of PCR-amplified DNA produced 100% $lacZ^+$ recombinant viruses when the homology was increased to two 50-bp

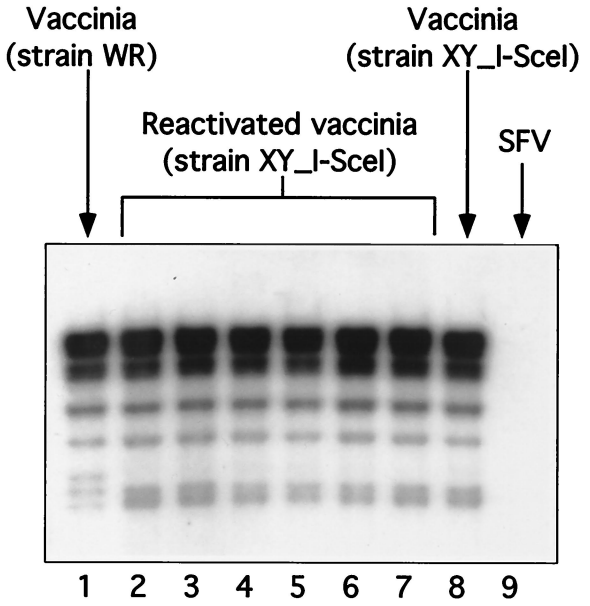

FIG. 2. Southern blot analysis of vaccinia virus genomes reactivated with a heterologous SFV helper virus. DNA was extracted from six different reactivated viruses, digested with *Hin*dIII, size fractionated by electrophoresis, and Southern blotted with a ^{32}P-labeled probe composed of purified vaccinia virus genomic DNA. All of the reactivated viruses that were recovered from cells transfected with XY-I-*Sce*IVV DNA (lanes 2 to 7) displayed a restriction fragment pattern characteristic of the parent strain XY-I-*Sce*IVV (lane 8). There was no evidence of deletions, fusions, or other rearrangements of the reactivated genomes. Lane 9 was loaded with *Hin*dIII-digested SFV DNA to show that SFV and vaccinia virus do not share a significant proportion of cross-hybridizing DNA fragments.

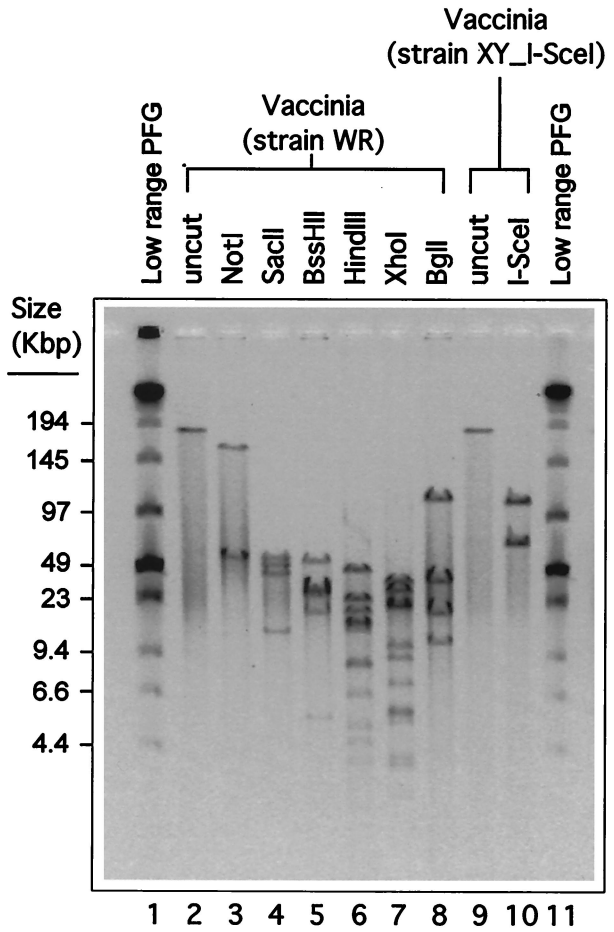

FIG. 3. Pulsed-field gel analysis of untreated and restriction enzyme-digested vaccinia virus DNA. DNA was extracted from vaccinia virus virions, size fractionated by pulsed-field agarose gel electrophoresis, and visualized by staining with ethidium bromide. Lanes 1 and 11, molecular size markers; lane 2, purified vaccinia virus DNA (strain WR); lanes 3 to 8, vaccinia virus DNA cut with the indicated restriction enzymes; lane 9, uncut vaccinia virus DNA (strain XY-I-*Sce*IVV); lane 10, XY-I-*Sce*IVV DNA cut with I-*Sce*I.

sequences (Fig. 6). We also confirmed that these results were not specific just for I-*Sce*I-cut vaccinia virus DNA. *Not*I cuts vaccinia virus strain WR only once in nonessential sequences (10). Similar yields of recombinant virus (4×10^4 PFU/μg, 22% recombinants) were obtained when *lacZ*-encoding PCR amplicons prepared with primers that added two 18-nucleotide sequence homologous to that flanking the *Not*I site were cotransfected into SFV-infected cells along with *Not*I-cut vaccinia virus DNA.

The production of $lacZ^+$ viruses need not have involved homology, since nonhomologous end-joining reactions could serve the same purpose and Southern blots would not be capable of discriminating between these two types of reactions. We tested the requirement for homology with a combination of I-*Sce*I-cut virus and the PCR amplicon originally designed to recombine with *Not*I-cut viral DNA. Such a combination of virus and DNA shows no end sequence homology beyond a few chance nucleotides. Cotransfecting this mixture of I-*Sce*I-cut vaccinia virus and PCR-amplified DNAs into SFV-infected cells yielded significant numbers of virus (5×10^5 PFU/μg), possibly by direct ligation, but only 0.08% were $lacZ^+$ recombinants. This low frequency of nonhomologous recombination is thus very similar to that observed previously in vaccinia virus-infected cells with transfected fragments of luciferase-encoding DNA (21).

Because the I-*Sce*I site is preceded by a T7 promoter and internal ribosome entry site derived from plasmid pTM3 (3, 11), the virus vector used in these reactions can also be used for the direct cloning and expression of recombinant proteins. DNA was extracted from vaccinia virus strain XY-I-*Sce*I, digested with I-*Sce*I, and cotransfected into SFV-infected cells along with a 760-bp promoterless DNA fragment encoding a GFP open reading frame. Two 20-nucleotide regions of homology permitted a recombination reaction that was expected to place the GFP gene under the regulation of the T7 promoter (Fig. 7A). Three days posttransfection, the resulting mixture of recombinant and nonrecombinant viruses was recovered and subsequently cocultivated for another 24 h on glass coverslips along with a helper virus expressing T7 RNA polymerase (5). Fluorescence microscopy was used to identify which infected cells produced recombinant green fluorescent protein. A significant portion (perhaps one-third) of the infected cells expressed GFP under these conditions (Fig. 7B).

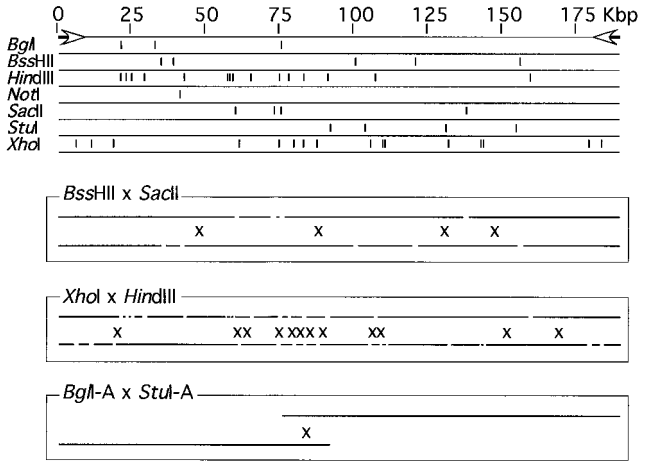

FIG. 4. Reactivation of vaccinia virus from transfected mixtures of overlapping DNA fragments. The upper portion of the figure shows a map of the restriction sites predicted and/or known to be present in vaccinia virus strain WR. It should be noted that our ATCC-derived stock of vaccinia virus strain WR lacks a *Sac*II site found at position 36041 in other stocks of strain WR as well as encoding a novel *Bss*HII site at position ≈41800. Arrows mark the terminal inverted repeats. Vaccinia virus DNA was digested with the indicated enzymes, and the three different pairwise combinations of fragments shown in the lower panels were transfected into SFV-infected cells. Vaccinia virus was recovered from cells transfected with a mixture of *Bss*HII- and *Sac*II-digested DNA but not from cells transfected with *Xho*I and *Hin*dIII fragments. The former reaction requires at least four recombination events to reassemble a complete vaccinia virus genome (x), and the latter requires at least 12 recombination events. Recombinant vaccinia viruses were also recovered from cells transfected with a mixture of *Bgl*I-A and *Stu*I-A fragments. In this case, the two largest restriction fragments were first purified of the remaining portions of the vaccinia virus genome by pulsed-field gel electrophoresis and recovered by electroelution.

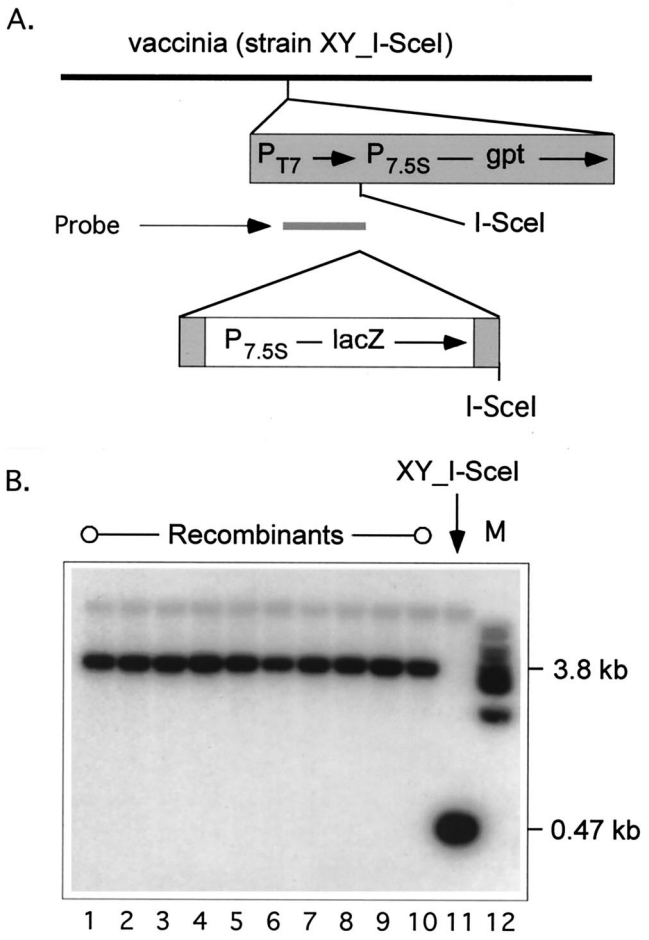

FIG. 5. Double-stranded break repair in SFV-infected cells. A recombinant vaccinia virus DNA was digested to completion with I-*Sce*I (Fig. 3) and purified by phenol extraction and ethanol precipitation. This DNA was transfected into SFV-infected cells along with a PCR-amplified DNA fragment encoding the *E. coli lacZ* gene and a vaccinia virus P7.5S promoter (17). The PCR primers used to amplify the *lacZ* cassette added two 18-bp terminal sequences identical to sequences surrounding the I-*Sce*I site (gray termini). Reactivated vaccinia viruses were plated on BSC-40 cells, and X-Gal was added to the medium to identify recombinants. DNA was extracted from $lacZ^+$ viruses and digested with *Avr*II and *Stu*I, and a Southern blot was performed to examine the DNA structure. Panel A shows the targeting strategy. Panel B shows a Southern blot probed with a DNA encompassing sequences upstream of and including the I-*Sce*I cleavage site. All of the $lacZ^+$ recombinant virus encoded a new 3.8-kbp restriction fragment (lanes 1 to 10). This fragment would be expected to form if recombinational repair reactions used DNA encoding the *lacZ* gene to repair I-*Sce*I-cut DNA.

Targeted deletion of a vaccinia virus restriction fragment. The efficiency of SFV-catalyzed reactivation methods suggested that the approach might also be used to assemble other modified forms of vaccinia virus genomes. To test this hypothesis, we investigated whether an 11.5-kbp fragment of the vaccinia virus genome could be deleted in a single step with a specially designed PCR amplicon. The experiment involved first digesting vaccinia virus DNA with *Bgl*I and then recovering the three largest DNA fragments from an agarose gel. The 11.5-kbp *Bgl*I-D fragment discarded at this stage has been shown previously to lack any genes essential for replication in culture (13). Four PCR primers, a vaccinia virus DNA template, two ordinary PCRs, and a subsequent PCR fragment fusion reaction were then used to prepare a 3.6-kbp linker DNA sharing end sequence homology with the two fragments flanking the missing *Bgl*I-D fragment but omitting nucleotides 21943 to 33500 (Fig. 8). This linker DNA (PCR1Δ) was then transfected into SFV-infected cells along with the other three *Bgl*I restriction fragments and a large PCR-amplified splice fragment (PCR5). PCR5 DNA was added to direct the recombinational repair of the double-stranded break separating the *Bgl*I-A and *Bgl*I-B fragments (Fig. 8). Reactivated viruses were then recovered, plaque purified, and characterized by PCR and Southern blotting (data not shown). In these experiments, the yield of reactivated virus was 3.8×10^3 PFU/μg, and 100% of the viruses (10 of 10) encoded a deletion of the expected number of bases. This yield of virus was very similar to that obtained by transfecting the three *Bgl*I restriction fragments into SFV-infected cells along with fragment PCR5 and a PCR fragment (PCR1) encoding all of the sequences deleted in PCR1Δ.

It should be noted that the viruses reactivated in the control reactions from mixtures of three *Bgl*I restriction fragments plus PCR1 and PCR5 DNA fragments are indistinguishable from the parental virus (vaccinia virus strain WR) because all of the DNAs were prepared with vaccinia virus WR reagents. To confirm that the genetic information incorporated between nucleotides 21943 and 33500 actually derived from PCR1 and not from a contaminating *Bgl*I-D fragment, we also prepared a PCR1 fragment with vaccinia virus strain Copenhagen DNA as the template. All of the virus reactivated from cells transfected with this $PCR1_{COP}$ DNA plus WR-derived *Bgl*I and PCR5 fragments bore an *Xba*I polymorphism indicative of the presence of *Bgl*I-D sequences originating in vaccinia virus strain Copenhagen (eight of eight viruses tested; data not shown). Besides demonstrating the purity of the mixture of strain WR-

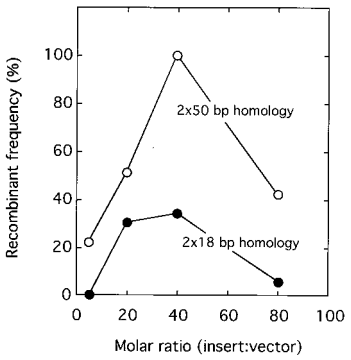

FIG. 6. Effect of DNA concentration and homology length on efficiency of recombinant virus production. SFV-infected cells were transfected with a mixture of DNAs comprising a fixed quantity of I-*Sce*I-cut vaccinia virus DNA and various amounts of PCR-amplified DNA encoding a P7.5S-*lacZ* expression cassette. Two different pairs of PCR primers were used to amplify the *lacZ*-encoding DNA, which added either two 18-bp or two 50-bp terminal sequences homologous to sequences flanking the I-*Sce*I-cut site (see Fig. 5). Reactivated viruses were subsequently diluted and plated, and the plaques were stained to detect β-galactosidase activity. A 30- to 40-fold molar excess of insert to vector molecules produced the greatest yield of recombinant virus with either substrate. Incorporating two 50-bp homologies permitted yields of ≈100% recombinant viruses.

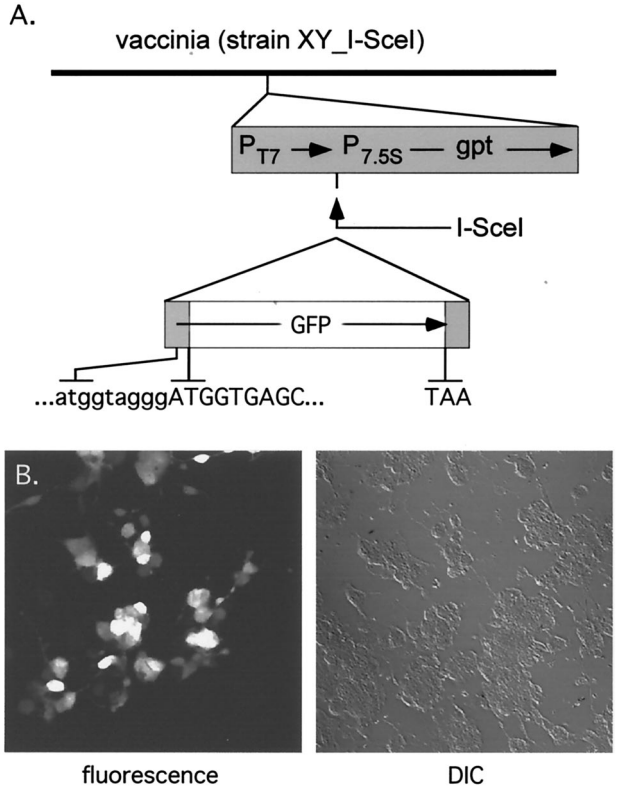

FIG. 7. Single-step construction of recombinant vaccinia viruses expressing green fluorescent protein. (A) SFV-infected cells were transfected with a mixture of DNAs consisting of I-*Sce*I-cut vaccinia virus DNA and PCR-amplified DNA encoding a GFP open reading frame. The PCR primers added two 20-bp terminal sequences homologous to sequences flanking the I-*Sce*I-cut site and created an in-frame fusion between the GFP open reading frame (capital letters) and a vector-encoded start codon (lowercase letters). (B) The resulting mixture of parental and recombinant viruses was recovered and titered and subsequently used to coinfect BSC-40 cells along with a helper virus expressing T7 RNA polymerase. Confocal fluorescence microscopy was used to detect GFP expression 24 h postinfection. These experiments used about 0.6 PFU of the reactivated virus mixture and about 6 PFU of VTF7.5 helper virus per cell.

derived *Bgl*I-A, *Bgl*I-B, and *Bgl*I-C fragments, this result illustrates how the method can be used to more precisely control the assembly of recombinant viruses from different viral strains.

In these experiments, we should also note that the PCR1Δ linker fragment was assembled from two separate DNAs, each encoding one of the two sequences homologous to those found flanking the *Bgl*I-D fragment. The assembly was accomplished with additional homologous sequences incorporated into the two central primers, and an in vitro PCR fusion reaction, to combine the 3.3-kbp (PCR1Δ-left) and 0.4-kbp (PCR1Δ-right) fragments into a single 3.6-kbp linker (PCR1Δ, Fig. 8). This step proved to be unnecessary, because deletion virus could also be recovered from SFV-infected cells that had been transfected with PCR1Δ-left, PCR1Δ-right, three *Bgl*I restriction fragments, and PCR5. However, requiring the additional recombinational exchange between DNAs sharing 30 nucleotide of sequence homology may have been responsible for reducing the yield of reactivated virus about fivefold (from 4×10^3 to 8×10^2 PFU/μg).

Recombinational substitution of essential portions of the vaccinia virus genome with large PCR amplicons. The studies described above showed that one can delete the *Bgl*I-D fragment and rescue the deficiency in reactivated viruses with a large PCR-amplified homolog. However, this is not a very rigorous test of the method because we used only a single fragment of DNA and *Bgl*I-D encodes no genes essential for virus replication. As a more demanding test of the approach, we examined whether portions of the virus encoding genes essential for growth in culture could also be PCR amplified and rescued into viable virus.

The first study examined whether a nearly complete set of overlapping PCR products could be assembled into a reactivated virus. We used Expand long-range PCRs to amplify a series of 12- to 22-kbp overlapping fragments spanning most of the vaccinia virus genome (Fig. 9). These fragments included the PCR1 and PCR5 fragments used previously. The lengths of the overlaps between different PCR fragments ranged from 0.3 to 9.3 kbp and were randomly determined by the manner in which a primer design program (Oligo 6) identified suitable primers. We did not try to amplify DNA located at the imme-

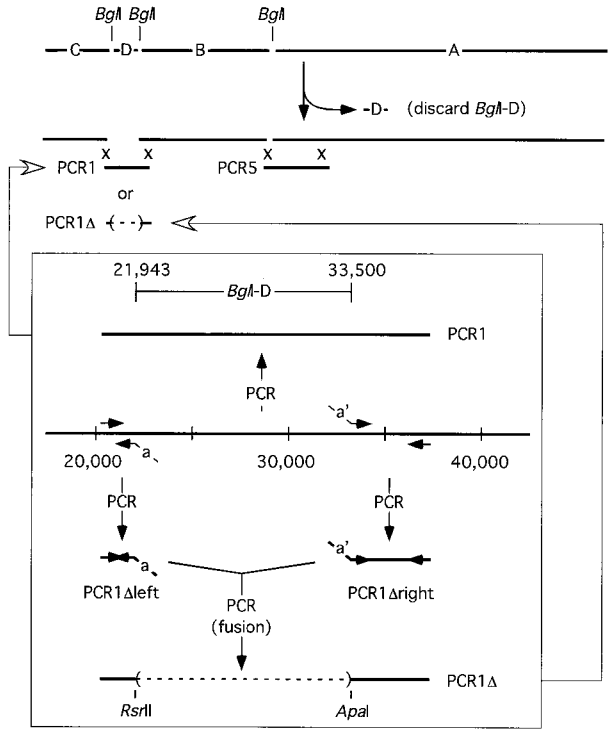

FIG. 8. Method used to construct a vaccinia virus deletion virus. Vaccinia virus DNA was digested with *Bgl*I, and the three largest fragments were separated from the smallest *Bgl*I-D fragment by pulsed-field gel electrophoresis. The three large fragments were recovered by electroelution and transfected into SFV-infected cells along with the PCR-amplified fragments indicated (PCR1 plus PCR5 or PCR1Δ plus PCR5). The ends of fragment PCR1Δ are identical to the ends of fragment PCR1, but an in vitro fusion reaction was used to join intermediate fragments PCR1Δ-left to PCR1Δ-right and thus eliminate most of the sequences that comprise the nonessential *Bgl*I-D fragment (boxed inset). Fragment PCR5 served to bridge the cut separating the *Bgl*I-A and *Bgl*I-B fragments.

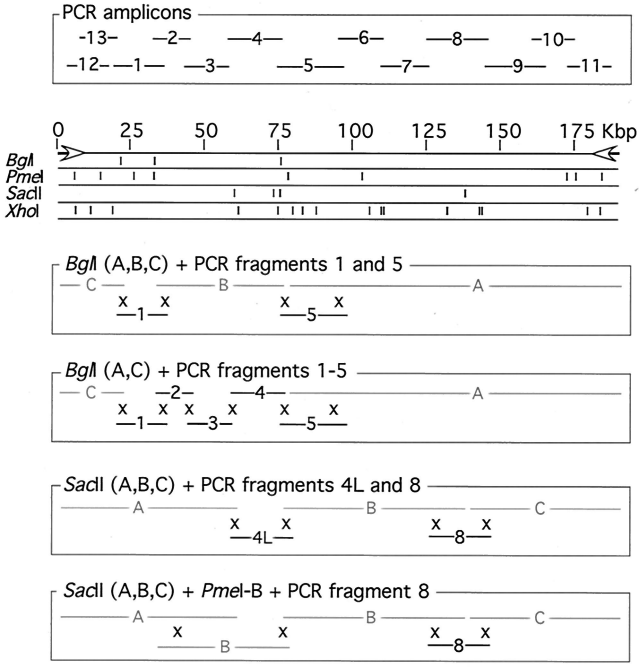

FIG. 9. Reactivation of vaccinia virus from cotransfected mixtures of PCR-amplified DNAs and vaccinia virus restriction fragments. The two top panels show the PCR fragments amplified for this study and their locations relative to a restriction map of vaccinia virus strain WR. The lower panels show the different combinations of PCR and restriction fragments tested as substrates in SFV-promoted reactivation reactions. No essential genes are encoded by fragment PCR1 (13). To create a sufficiently large overlap between PCR-amplified and *Sac*II-digested DNAs, the 18.6-kbp PCR4 fragment (top panel) was replaced with a larger 21.3-kbp PCR4L fragment (Table 1). Genetic analysis and gene ablation studies have shown that PCR4L encodes numerous essential genes. These include but are not limited to a portion of I3L (14), I8R (6), G2R (7), G4L (20), and G8R (22).

diate ends of the genome because of anticipated difficulties with using PCR to reproduce such telomeric features as hairpins and mismatched bases. Instead, vaccinia virus genomic DNA was digested with *Xho*I, and the resulting ≈5-kbp restriction fragments were isolated from agarose gels. All of these DNA fragments were combined in the appropriate molar ratios and cotransfected into SFV-infected BGMK cells, and any resulting virus was rescued by replating on BSC-40 cells. Despite repeated attempts, these experiments failed to generate reactivated virus.

It was of some concern that all of the DNAs used in these experiments had been purified from agarose gels, because this method can introduce contaminants into DNA substrates. To show that no inhibitory contaminants were present, we added *Xho*I-cut vaccinia virus DNA to the mixture and cotransfected this pool of substrates into SFV-infected cells. This mix of natural and synthetic DNA fragments permitted recovery of virus with a yield of $\approx 2 \times 10^3$ PFU/μg.

To gain some understanding as to what other factor(s) might have prevented these experiments from working, we examined whether progressively less complex mixtures of natural and PCR-amplified vaccinia virus DNAs could be recombined and reactivated in SFV-infected cells. Noting that a mixture of PCR1 and PCR5 fragments along with *Bgl*I-A, -B, and -C restriction fragments permitted recovery of reactivated virus, we tested whether virus could also be rescued from a combination of just the *Bgl*I-A and *Bgl*I-C restriction fragments plus PCR fragments 1 to 5 (Fig. 9). Again, no reactivated viruses were recovered with this strategy. Finally, we further simplified the experiment so that only a single large and yet essential PCR fragment had to be rescued into the vaccinia virus genome. Several different regions of the vaccinia virus genome were examined, and we were able to reproducibly recover recombinant and reactivated virus with at least one particular combination of natural and PCR-amplified DNA.

These studies used the three largest vaccinia virus *Sac*II restriction fragments and PCR fragments 4L and 8 (Fig. 9). PCR4L (a slightly larger derivative of PCR4) shared 3.3 and 2.5 kbp of flanking sequence homology with the adjacent *Sac*II fragments and spanned the genetic interval encompassing genes I3L to L4R. It thus encoded many genes known to be essential for viral growth and assembly (6, 7, 14, 20, 22). PCR8 served only as a recombinational bridge between the *Sac*II-B and *Sac*II-C fragments (Fig. 9). When SFV-infected BGMK cells were transfected with this DNA mixture, we obtained yields of recombinant virus that were essentially identical to those obtained when a control *Pme*I-B restriction fragment was used instead of PCR4L (8.5×10^5 versus 8.2×10^5 PFU/μg, respectively).

Southern blots were later used to confirm that all (10 of 10) of the viruses recovered and tested were genetic hybrids. To show this, we assembled a recombinant virus with a heterologous combination of WR *Sac*II restriction fragments and Copenhagen "templated" $PCR4L_{COP}$ DNA and used restriction fragment polymorphisms to identify the origins of different parts of the resulting virus. A probe targeting *Sac*II-D sequences detected a *Hinc*II site polymorphism in the reactivated viruses characteristic of strain Copenhagen, while a probe targeting the *Bgl*I-D region (Fig. 9) detected an *Xba*I site polymorphism characteristic of strain WR (data not shown). We concluded that one can rearrange essential portions of the vaccinia virus genome with these methods, but probably only a single amplicon at a time.

DISCUSSION

These experiments show that SFV, like fowlpox virus, can be used to rescue and reactivate vaccinia virus in cells transfected with vaccinia virus DNA. However, an important difference is that this seems to be by far the most efficient in vitro heterologous poxvirus reactivation reaction described to date, and this contention is supported by direct comparisons. These show that SFV-infected cells yield ≈100-fold more reactivated vaccinia virus than do fowlpox-infected cells (M. Merchlinsky, personal communication). This increase in efficiency offers significant experimental advantages, but the numbers still suggest that only a small proportion of input genomes contribute to the pool of reactivated viruses that one can eventually recover from SFV-infected cells. Perhaps this is not too surprising because, during the early steps in the process of virus rescue, a mixture of virus proteins would be expected to arise that might well interfere with the activity of multicomponent protein complexes or the assembly of virus capsids.

Either mixed infections of orthopoxviruses, leporipoxvi-

ruses, and avipoxviruses are thus able to segregate orthologous proteins into properly distinct protein complexes, or the architecture of these complexes is sufficiently flexible to accommodate proteins typically showing only 60 to 80% amino acid identity. The observation that SFV seems to reactivate vaccinia virus much better than fowlpox virus suggests that the latter process may operate under these experimental conditions, since SFV proteins might be more compatible with vaccinia virus proteins, given the closer evolutionary relationship. SFV-infected cells also catalyze very high levels of nonspecific DNA replication (1) and recombination (4, 12), and these reactions may be another factor contributing to the efficiency of the overall process by more efficiently amplifying and repairing transfected vaccinia virus genomes.

One advantage of using fowlpox helper viruses is that the genetic distances which separate avipoxviruses from orthopoxviruses minimize the risk that mixed infections will produce intertypic virus recombinants. Leporipoxviruses and orthopoxviruses also appear to have been sufficiently isolated by evolutionary processes to prevent SFV from recombining with vaccinia virus. All of the viruses that we have rescued to date seem to grow normally, and although we have not pursued an exhaustive screen for intertypic recombinants, no such viruses were detected with either Southern blots (vaccinia virus) or genetic methods (myxoma virus).

This failure to recover intertypic recombinants probably depends upon two favorable factors. First, hybrid viruses would probably exhibit growth deficiencies of various severities that would reduce their abundance in mixed populations of replicating viruses. Second, when one compares DNA sequences, about one-quarter of the bases differ between even the most closely related virus genes (probably SFV S068R and vaccinia virus J6R), and one cannot detect even this limited homology with Southern blots (Fig. 2). This is probably insufficient sequence identity to permit efficient recombination, and collectively these two constraints would compromise the recovery of intertypic recombinant viruses. We believe that, as a method of genetic isolation, the use of helper viruses like fowlpox and SFV to reactivate orthopoxviruses is preferred to the use of homologous psoralen-inactivated orthopoxviruses (19). Heterologous helper viruses seem to be genetically inert, while chemically inactivated viruses could contribute heavily damaged DNAs to a pool of molecules interacting in a highly recombinogenic environment.

Several practical uses for the method have been identified in this study which exploit the high-frequency recombination and nonspecific DNA replication systems we characterized previously. In particular, one can utilize fortuitously located restriction sites and PCR-generated linker fragments to create targeted deletions of nonessential portions of poxvirus genomes. One could presumably continue with this process in a stepwise manner by taking further advantage of existing as well as newly introduced restriction sites to create a succession of progressively smaller viruses. Appropriately modified viruses can also be used to facilitate the conditional expression of recombinant proteins. The production of recombinants is most efficient when rather long patches of flanking homology are used to target the insert into the double-stranded break (two 50-bp patches, Fig. 6), but even two 18-bp patches of homology can yield recombinants at frequencies of up to 30%. In this regard, the effect of homology length on reaction efficiency is qualitatively similar to that we characterized previously in vaccinia virus-infected cells (12, 21), although the different selection methods render absolute comparisons difficult. SFV-promoted recombination and reactivation reactions are sufficiently efficient that one can directly detect the expression of vaccinia virus-encoded recombinant green fluorescent protein without further selection, propagation, or plaque purification of the recombinant virus (Fig. 7).

Despite the high recombination frequencies detectible in SFV- and vaccinia virus-infected cells, it seems likely that a "numbers game" ultimately places practical limits on the capacity of these systems to generate recombinant viruses. These limits are of little concern where a simple double strand break repair reaction is used to insert one piece of DNA into a cut vector with reactions of the type illustrated in Fig. 5 and 7. However, as the number of exchanges increases, the overall yield of reactivated virus is expected to decrease in a manner that depends upon the efficiency of each component recombination reaction. This is best illustrated by considering the impact of each additional recombination step occurring with an efficiency near 50% versus only 20%. The overall yield of virus is crudely expected to follow the relationship $N = N_o \times E^x$, where N is the overall yield of virus (PFU per microgram), N_o is the maximal yield possible with intact transfected DNA, E is the average efficiency of each component recombination event, and x is the number of exchanges. With a typical maximal yield of approximately $N_o = 10^6$ PFU/μg and the lowest practical yield $N = 1$ PFU/μg, solving for x suggests that virus should be recoverable if the number of exchanges ranges from 8 ($E = 0.2$) to 20 ($E = 0.5$).

These values do seem to be useful working limits for this system. For example, very high yields of virus were obtained with mixtures of *Bss*HII- and *Sac*II-cut vaccinia virus DNA (Fig. 4) in a reaction requiring only four exchanges and involving extensive (i.e., efficiently recombined) overlaps. Conversely, virus could not be recovered from cells transfected with a mixture of *Xho*I- and *Hin*dIII-cut vaccinia virus DNA. In this situation, at least 12 exchanges are required, and some of the short overlaps between fragments (as little as 0.2 kbp) might also be expected to reduce the average recombination efficiency.

A rather surprising feature of this process is that it can be used to reactivate vaccinia viruses from transfected mixtures of virus restriction fragments and large PCR-amplified portions of the virus genome. It is surprising because it is expected that viruses produced by this method would encode multiple new mutations due to the poor fidelity of the DNA polymerases used in the PCR. These error frequencies vary from 2.6×10^{-5} (for *Taq* polymerase) to 8.5×10^{-6} (Roche high-fidelity PCR system). Using the Roche long-template PCR systems, we are exhibiting an accuracy that probably falls somewhere between these two bounds. If one used 20 PCR cycles to create a pool of ≈17-kbp PCR products, each DNA would then bear an average of 3 or 13 mutations per molecule if these DNAs were amplified with high-fidelity and *Taq* polymerases, respectively. Only ≈5% of the 17-kbp molecules amplified with high-fidelity proofreading enzymes would be expected to be free of errors, and essentially none of the DNAs amplified with *Taq* polymerase would be error free. Most of these mutations would be

silent, and so these errors might not be enough to prevent the recovery of recombinant viruses with a single large PCR amplicon encoding numerous essential virus genes. However, it becomes more and more unlikely that one could reactivate a virus from mutation-free PCR amplicons as the number of such fragments increases. This fact may explain why one cannot reactivate virus from multiple pieces of PCR-amplified DNAs and suggests that even the most efficient poxvirus reactivation methods could not provide a facile route for reactivating orthopoxviruses if the only available source of virus DNA was PCR-amplified or chemically synthesized materials.

In conclusion, we have shown that the DNA replication and recombination systems found in cells infected with replicating poxviruses (1, 12) probably also play an important role in catalyzing virus reactivation reactions. The unusually hyperrecombinogenic environment created in SFV-infected cells can also be exploited to provide a simple method for rearranging the structure of poxvirus genomes and might ultimately even provide a novel way of securely archiving orthopoxviruses in an inert form. One could envision purifying the virus DNA, cutting it with different restriction enzymes, and storing different digests in separate locations. This process would address public concerns about the storage of variola virus by rendering the stocks noninfectious and difficult to reactivate unless one had access to both pools of DNA.

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